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Female mice respond differently to costly foraging *versus* food restriction

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SUMMARY

Experimental manipulation of foraging costs per food reward can be used to study the plasticity of physiological systems involved in energy metabolism. This approach is useful for understanding adaptations to natural variation in food availability. Earlier studies have shown that animals foraging on a fixed reward schedule decrease energy intake and expenditure. However, the extent to which these changes depend on decreased food intake or increased foraging costs *per se* has never been tested. We manipulated foraging costs per food reward in female Hsd:ICR(CD-1) laboratory mice, comparing animals faced with low (L) and high (H) foraging costs to non-foraging animals receiving a food restriction (R) matched to the intake of H animals. Mice in the H group ran as much as L mice did but ate significantly less. They concurrently reduced daily energy expenditure and resting metabolic rate, decreased the size of major metabolic organs and utilized body fat stores; mass-specific resting metabolic rate did not differ between groups. We found evidence that these alterations in energy balance may carry fitness costs. As a secondary response to our experimental treatment, H females and, eventually, some R females ceased to show signs of estrous cyclicity. Surprisingly, results of an immune challenge with keyhole limpet hemocyanin showed that primary immune response did not differ between L and H groups, and was actually higher in R mice. Our results demonstrate that high foraging costs *per se* – the combination of high activity and low food intake – have pronounced physiological effects in female mice.

Key words: foraging costs, food restriction, workload, daily energy expenditure (DEE), resting metabolic rate (RMR), allocation trade-offs.

INTRODUCTION

Energy balance is a key mediator of individual survival and reproductive output in free-living animals, which makes energy a useful currency for understanding life history trade-offs (Drent and Daan, 1980; Ricklefs and Wikelski, 2002; Speakman, 1997; Stearns, 1992). Several environmental, physiological and behavioral factors may influence patterns of energy intake and expenditure in free-living animals (Speakman, 2000). Among these, there has been limited but intriguing evidence suggesting that environmental quality influences both resting metabolic rate (RMR) and daily energy expenditure [DEE (Speakman et al., 2003)]. One attractive hypothesis is that poor environments may force animals to expend additional energy as a result of the increased cost of foraging (Wiersma et al., 2005). However, such a functional link is far from being established. An experimental approach is useful to gain further insight into the manner in which foraging costs influence patterns of energy allocation.

Several studies have manipulated foraging costs per reward in the laboratory using a work-for-food design. In rodents, wheel running is typically used as a proxy for foraging activity (Perrigo, 1987; Perrigo and Bronson, 1983; Perrigo and Bronson, 1985; Vaanholt et al., 2007), whereas studies in avian species have required animals to fly short distances, hop on perches or sift through chaff to receive food (reviewed by Wiersma and Verhulst, 2005). Animals facing elevated foraging costs increase their activity or keep activity constant (Vaanholt et al., 2007; Wiersma and Verhulst, 2005). Food

intake and DEE are decreased when a fixed reward schedule is used (Bautista et al., 1998; Day and Bartness, 2001; Deerenberg et al., 1998; Perrigo, 1987; Vaanholt et al., 2007) but may be elevated when rewards are unpredictable (Bautista et al., 1998; Wiersma et al., 2005).

Various types of changes occur in response to manipulation of foraging costs. Animals typically alter their time–activity budgets (Deerenberg et al., 1998; Perrigo, 1987), utilize stored energy (Day and Bartness, 2001; Vaanholt et al., 2007) and reduce whole body mass and organ size (Vaanholt et al., 2007). Reductions in resting metabolic rate are typical (reviewed by Wiersma et al., 2005). Even more extreme savings may occur through hypothermia, which has been observed in starving animals maintained on low levels of nutrition (e.g. Daan et al., 1989; Gelezen et al., 2006; Hudson and Scott, 1979) and may also be possible under elevated foraging costs (Perrigo and Bronson, 1983; Vaanholt et al., 2007).

In addition to changes in patterns of energy use, high foraging costs have secondary physiological consequences which could compromise fitness and survival prospects of free-living animals. Most small mammals are sensitive to food availability in scheduling their reproductive effort (Wade and Schneider, 1992), and alterations in energy balance may reduce fertility and breeding success (Johnston et al., 2006). Perrigo (Perrigo, 1987) found that high foraging costs are detrimental to breeding success of two species of wild mice (*Mus musculus* and *Peromyscus maniculatus*). Along with reductions in total expenditure, animals faced with high

foraging costs reallocate energy away from somatic repair [e.g. feather re-growth (Wiersma and Verhulst, 2005)], protection from cellular damage by free oxygen radicals (Wiersma et al., 2004), and immune function (Deerenberg et al., 1997). Trade-offs of this type can affect mortality risk and rates of senescence.

Work-for-food experiments are a tool which has been used to mimic natural variation in foraging costs. This design simultaneously exposes animals to the dual energetic challenges of forced activity and reduced food intake. It has thus far remained unclear to what extent changes in physiological indicators of energy balance (e.g. body composition and metabolic rate) are the combined result of increased rates of energy turnover on the one hand and of food restriction on the other. To address this problem, we set out to explicitly separate these effects. We manipulated female laboratory mice by exposing them to low and high foraging costs and compared the latter to a group of inactive animals receiving a food ration matched to the intake of animals with high foraging costs. We measured activity, food intake, energy metabolism, body mass and composition, as well as secondary responses in estrous cyclicity and in primary immune response to a novel antigen challenge. Our hypothesis was that effects of high foraging costs are due to the combined effect of food restriction and high energy turnover. Therefore, we predicted that effects such as decreased immune response, and loss of ovarian cyclicity would be more dramatic in animals facing high foraging costs than either other group.

MATERIALS AND METHODS

Animals and conditions

A total of 24 female mice (*Mus musculus domesticus* L.) of the outbred Hsd:ICR(CD-1) strain, bred at our facilities at the Biological Center in Haren, The Netherlands, were used in the experiment; founders of this population were obtained from Harlan, France in 2005. At the age of 4 months animals were individually housed in standard cages (Macrolon type II long 33×15×13 cm, UNO Roestvaststaal BV, Zevenaar, The Netherlands) without running wheels. All mice were kept in the same room at 21±1°C on a 12 h:12 h light:dark cycle (lights on 04:00 GMT+1 h) and had *ad libitum* access to water and food (standard rodent chow: RMH-B/2181, Arie Block BV, Woerden, The Netherlands). Mice were abdominally implanted with 1.2 g temperature transmitters (Series 3000 XM-FH Mini Mitter, Bend, OR, USA) under surgical anesthetic and were allowed to recover for one week in their home cages before the start of the experiment. Temperature data are not presented here, and body mass data were corrected for the mass of implanted transmitters. All procedures concerning animal care and treatment were in accordance with the regulations of the University of Groningen's ethical committee for the use of experimental animals (license DEC 4321).

Experimental design

At the start of the experiment, animals were assigned to three experimental groups ($N=8$ per group): (group 1) L, mice that faced low foraging costs; (group 2) H, mice that faced high foraging costs; (group 3) R, mice that did not have to forage for food, but were each pair-fed with a mouse from the H group. At this time L and H mice were housed in Plexiglas cages (20×20×30 cm) fitted with plastic running wheels (diameter 14 cm; code 0131 Savic®, Kortrijk, Belgium). R mice remained in standard cages without wheels. The study was

divided into three phases: baseline, training and workload phases. The start of the training phase was termed experimental day 0. Time notation followed this convention throughout. Body mass and food intake (to the nearest 0.1 g) were measured daily throughout the study, starting 1–2 h prior to lights off so that the disturbance coincided roughly with activity onset. This was also the time at which the R mice received their food ration based on the food intake of the H mice over the previous day. The weighing sequence was rotated daily. After weighing, we hand-sifted bedding to count uneaten food blocks or pellets, which were always removed. Running-wheel activity [RWA; in number of revolutions (revs)] was logged in 2 min time intervals (bins) using a computerized event recording system. We calculated travel distances in km day^{-1} and estimated time spent running (h day^{-1}) by counting the number of 2-min bins with a value of >0. Maximum and mean running speeds were estimated from the highest number of revolutions in a 2-min bin and from the mean number of revolutions across all non-zero bins, respectively. Activity of R mice housed without wheels was not measured.

In the baseline phase (days –27 to –1), all mice were kept on *ad libitum* food. Individual baseline reward rates (in revs pellet^{-1}) were calculated by dividing mean RWA (revs day^{-1}) by gross energy intake (kJ day^{-1}) for each mouse over days –14 to –1 of this phase. This measure was then adjusted for the manufacturer's energy content of the rodent blocks given during the baseline phase and the precision pellets which would later be used during the training and workload phases (Table 1).

In the training and workload phases, all animals were switched to a diet of 45 mg precision food pellets (TestDiet 5TUM/PJAI, Sandown Chemicals, Hampton, Surrey, UK). Cages of L and H mice were connected to food dispensers (Med Associates Pellet Dispenser ENV-203, Sandown Scientific, Hampton, UK) that delivered food pellets on a fixed reward ratio (as described above) and were linked to a steering computer (Series 3 Programmable Controller, General Electric). On average, mice had to run $130\pm10 \text{ revs pellet}^{-1}$ ($\sim 17000 \text{ revs day}^{-1}$) to obtain their baseline food intake. Foraging costs of the L mice remained constant at their individual reward rate during the training and workload phases (days 0–40). Thus, when running the same amount as during the Baseline phase, they would have received an amount of food similar to their mean intake during that phase. In practice, L mice ran more during the training and workload phases than during the baseline phase and received more pellets than they ate each day. In the H group, foraging costs per reward increased over the training phase: foraging costs were raised by 10% of the individual baseline every other day, until they had doubled. Foraging costs then remained constant at 200% of baseline, marking the start of the workload phase (day 21). Mice in the R group did not forage to obtain food, but were pair-fed with animals from the H group: each R-group mouse received a daily ration of precision pellets matched

Table 1. Food types and estimated energetic content

	Rodent blocks	Precision pellets
Product name	RMH-B/2181 (<i>Arie Block</i>)	5TUM/PJAI (<i>TestDiet</i>)
Experimental phases	Baseline	Training and workload
Energy (composition) (kJ g^{-1})	16.10	13.40
Energy (bomb calorimetry) (kJ g^{-1})	18.42	18.16
Energy (composition) is based on manufacturer's information, given the decimal fractions of protein, fat and carbohydrate and standard energetic values.		
Energy (bomb calorimetry) is based on our own measurements using a ballistic bomb calorimeter (Miller and Payne, 1959); this estimate includes a thermochemical value of indigestible as well as digestible contents.		

to the previous day's intake of an individual H mouse (i.e. they were 'yoked controls').

Estrous cyclicity

Vaginal cytology was scored after daily weighing from day -27 to 28. A vaginal swab was taken from each animal (while gently holding it by the tail) using a paintbrush dampened with distilled water. Cells were smeared onto a clean glass slide and stained with a drop of Methyl Blue dye (S. Johnston, personal communication). A single observer blind to time and treatment visually scored slides with a light microscope at 100 \times magnification. Following Miller et al. (Miller et al., 2004), the cycle phase was identified based on the proportion of leukocytes, nucleated epithelial cells, and cornified epithelial cells counted in >100 cells per smear. An animal was considered fertile in the baseline phase if it entered estrous ($\geq 50\%$ cornified cells per smear) on at least three occasions between day -27 and -1, and at least once between day -7 and -1. For statistical analyses, the day with the highest percentage of cornified cells measured in a 1-week period (see Data analysis below) was used as an estrous score.

Metabolic measurements

In the workload phase [days 25–28 (± 1 day)] daily energy expenditure (DEE; kJ day $^{-1}$) of animals in their home cages was estimated using the doubly labeled water method [DLW (Lifson and McClintock, 1966; Speakman, 1997)]. The protocol followed Vaanholt et al. (Vaanholt et al., 2007). Mice were weighed to the nearest 0.1 g, briefly restrained by the scruff of the neck, and injected i.p. with ~ 0.1 g of enriched water (37.6% ^2H and 60.6% ^{18}O). The precise dose injected was quantified by weighing syringes to the nearest 0.0001 g before and after injection. After a 1 h equilibration period (Król and Speakman, 1999) animals were bled at the tail tip, and an initial blood sample (15 μl) was collected in duplicate glass capillary tubes, which were immediately flame-sealed with a propane torch. Mice were then returned to their home cages. Forty-eight hours after the initial sample (Speakman and Racey, 1988), a final blood sample was collected in triplicate following the same procedure.

After collecting final blood samples for DLW, metabolic rate was measured overnight in an open-flow respirometry system (Oklejewicz et al., 1997; Vaanholt et al., 2007). Mice were placed in transparent Plexiglas chambers (15 \times 10 \times 10 cm) with a slice of apple and some home-cage bedding. They were fed a ration of the same type and quantity of food they consumed the previous day. Measurements were made for ca. 23 h under the same temperature and photoperiod conditions as in the experimental room. We used an eight-channel system which sampled each mouse over a 1 min interval once every 10 min and recorded differentials in gas concentrations between excurrent chamber air and reference air (drier: 3 Å molecular sieve drying beads, Merck, Darmstadt, Germany). O_2 consumption (\dot{V}_{O_2} ; ml h $^{-1}$; Servomex Xentra 4100 paramagnetic analyzer, Crowborough, UK) and CO_2 production (\dot{V}_{CO_2} ; ml h $^{-1}$; Servomex 1440 infrared analyzer) were measured simultaneously. Inlet airflow was set at 20 l h $^{-1}$ (Brooks Type 5850 mass flow controller, Rijswijk, The Netherlands). We calculated the respiratory quotient (RQ) as $\dot{V}_{\text{CO}_2}/\dot{V}_{\text{O}_2}$. Metabolic rate (MR; kJ h $^{-1}$) was calculated from the formula (Romijn and Lokhorst, 1961):

$$\text{MR} = 16.18 \times \dot{V}_{\text{O}_2} + 5.02 \times \dot{V}_{\text{CO}_2}. \quad (1)$$

Resting metabolic rate (RMR; kJ h $^{-1}$) was estimated as the lowest value of a 20 min running mean of MR (typically three measurement

points) and later expressed as kJ day $^{-1}$. We also estimated the average daily metabolic rate (ADMR; kJ day $^{-1}$) for animals in the metabolic chambers.

Mass spectrometry

Determinations of $^2\text{H}:^1\text{H}$ and $^{18}\text{O}:^{16}\text{O}$ ratios in blood samples were performed by mass spectrometry at the University of Groningen's Center for Isotope Research (Visser and Schekkerman, 1999). Blood samples were prepared by microdistillation in a vacuum line, first heating the broken tubes and then cryogenically trapping the emerging water vapor with liquid nitrogen (Nagy, 1983). Water samples were stored and then automatically injected into a Hekatech high temperature pyrolysis unit (Gehre et al., 2004), in which the injected water was reacted with glassy carbon. The resultant H_2 and CO gases, emerging into a continuous He flow-through system, were then led through a GC column to separate the two gases in time and finally fed into a GVI Isoprime isotope ratio mass spectrometer for the analysis of $\delta^{18}\text{O}$ and $\delta^2\text{H}$. Measurements were corrected for memory effects using an algorithm similar to the one described by Olsen et al. (Olsen et al., 2006). At least three internal water standards chosen to cover the entire enrichment range of the blood samples were prepared and analyzed following the same methods. We measured samples in duplicate unless a flaw was detected in the flame-sealing step. Typical relative duplo differences were below 2.5% for $\delta^2\text{H}$, and 1% for $\delta^{18}\text{O}$. If differences exceeded 3% we critically examined the data and omitted the aberrant value, performing further calculations on a single replicate. Otherwise, duplicate values were averaged.

Initial isotope dilution spaces (mol) were calculated by the intercept method (Coward and Prentice, 1985); total body water was converted to grams using a molecular mass of 18.020 for body water, and expressed as a percentage of body mass. The rate of CO_2 production (r_{CO_2} ; moles day $^{-1}$) was calculated using Speakman's single-pool model equation 7.17 (Speakman, 1997):

$$r_{\text{CO}_2} = N/2.078 \times (k_o - k_d) - 0.0062 \times N \times k_d, \quad (2)$$

where N is the size of the body water pool (mol), k_o and k_d are the respective fractional turnover rates (per day) of ^{18}O and ^2H (Lifson et al., 1955), which were calculated against background concentrations (Speakman and Racey, 1987) and the individual-specific initial and final ^{18}O and ^2H concentrations (Speakman and Król, 2005). Finally, r_{CO_2} was converted to energy expenditure assuming an energetic equivalent of 22.0 kJ l $^{-1}$ CO_2 based on the Weir equation (Weir, 1949) and average respiratory quotient from our respirometry measurements (0.95).

Immune challenge

On day 39 (± 1 day), primary immune responsiveness was assayed by challenging animals with a novel antigen, keyhole limpet hemocyanin (KLH). After weighing, each mouse was injected s.c. with 0.1 ml of 0.9% sterile saline containing 150 μg KLH (Calbiochem, Merck KGaA, Darmstadt, Germany; #374811, lot #B304050). Blood samples were taken from the tail tip at 5 and 10 days after injection to measure anti-KLH immunoglobulin (IgG) production. Samples of $\sim 100 \mu\text{l}$ were collected in unheparinized glass capillary tubes and allowed to clot on ice. After removing clots, samples were centrifuged at 2500 g for 1 h at 4 $^{\circ}\text{C}$. Serum was aspirated and stored it at -80°C until analysis with an enzyme-linked immunosorbant assay for anti-KLH IgG [analyzed at Indiana University by G. D. following Demas et al. (Demas et al., 1997)]. Briefly, 96-well microtiter plates were coated with antigen by

incubating overnight at 4°C with 0.5 mg ml⁻¹ KLH, washed, and then blocked with 5% nonfat dry milk overnight at 4°C to reduce nonspecific binding. Plasma samples were diluted 1:20. Pilot data showed that peak immune response occurred between day 10 and 15 post-injection; therefore, we only analyzed samples taken on day 10. Positive control samples (pooled plasma from mice previously determined to have high anti-KLH antibodies) and negative control samples (pooled sera from KLH-naïve mice) were also added in duplicate to each plate. Secondary antibody [alkaline phosphatase-conjugated anti-mouse IgG diluted 1:2000 with phosphate-buffered saline plus 0.1% Tween 20 (PBS-T; Cappel, Durham, NC, USA)] was added, and plates were read with a 409 nm filter following the addition of the enzyme substrate *p*-nitrophenyl phosphate (Sigma Chemical, St Louis, MO, USA). Mean optical densities (OD) are expressed as a percentage of the plate positive control OD for statistical analyses.

Body composition

Ten days after KLH injection, animals were sacrificed for carcass analysis between 13:00 h and 17:00 h (GMT+1 h). Animals were killed by CO₂ inhalation and immediately decapitated. Bodies were dissected to separate organs, skin and the musculoskeletal system, and weighed to the nearest 0.0001 g. Samples were stored at -20°C until analysis. We determined dry and lean dry organ masses by drying to constant weight at 103°C [European Standard Protocol ISO 6496-1983(E)] followed by fat extraction with petroleum ether (Boom BV, Meppel, The Netherlands) in a Soxhlet apparatus.

Data analysis

We tested two experimental hypotheses: that high foraging costs *per se* alter behavior and physiology of female mice (H_{A1}: H females differ from L females), and that high foraging costs induce quantitatively different responses than food restriction alone (H_{A2}: H females differ from R females). Because L and R mice differed in two respects – both foraging activity and feeding regime – we could not make *a priori* hypotheses about the differences between them and did not statistically compare these groups. We tested responses in the workload phase using unpaired *t*-tests. Some baseline-phase parameters differed between groups, but including baseline phase parameters as model covariates never yielded different results in *t*-tests (ANCOVA; results not presented). Some additional comparisons used general linear models (GLM).

Three animals died or became ill and were retrospectively excluded from the study. Sample sizes for most analyses were therefore L=8, H=6 and R=7. Because of technical problems, we further restricted analyses of DLW data to L=7, H=6 and R=5; for consistency, we used the same subset of mice for other analyses related to energy metabolism. Data were analyzed using Statistica v. 6.1 (StatSoft, Inc., Tulsa, OK, USA). We checked data for normality and arcsin-transformed proportional measures to sin⁻¹(\sqrt{y}) before analysis (arcsin-sqrt). Two-tailed *P*-values of ≤ 0.05 were considered statistically significant. Because most organ masses were correlated within individuals, we analyzed body composition using a principal components analysis [PCA; after Selman et al. (Selman et al., 2002)]. This procedure addresses the problem of multiple statistical comparisons on correlated data by creating a smaller number of uncorrelated response variables (principal components) which account for the maximum amount of variation in the data. We applied Varimax normalized rotation to factors with Eigenvalues ≥ 1 and performed statistical comparisons on these rotated factors.

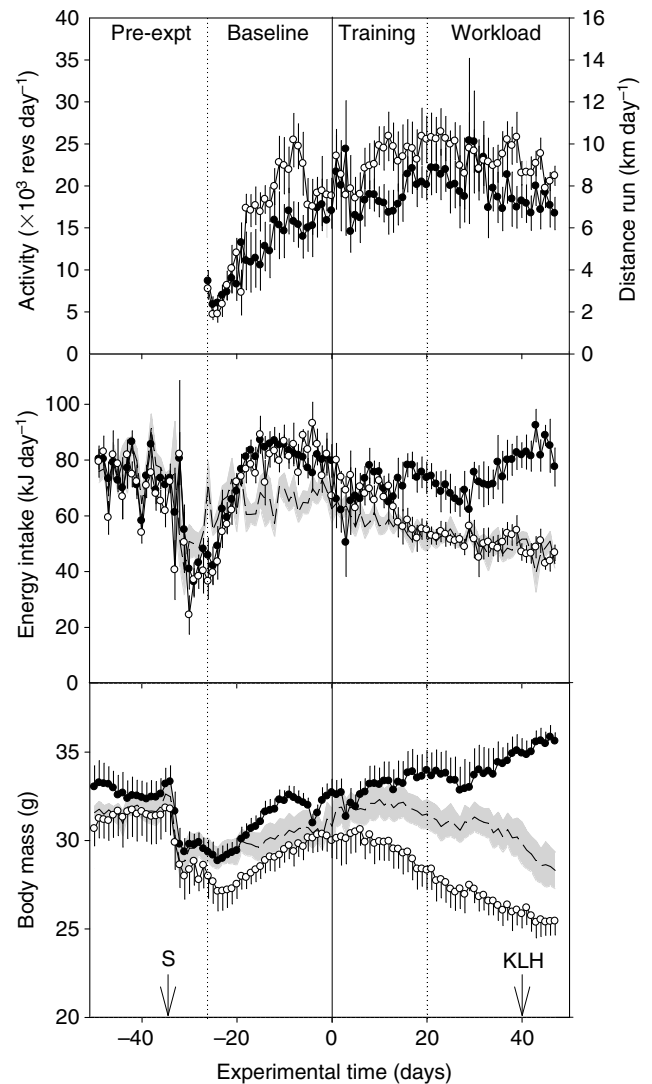


Fig. 1. Development of wheel-running activity (revs day⁻¹ and km day⁻¹), energy intake (kJ day⁻¹) and whole body mass (g) of female mice facing low foraging costs (L; filled circles), high foraging costs (H; open circles) and restricted feeding without foraging effort (R; gray shading). Error bars show the standard error of the mean. Temperature transmitters were surgically implanted (S) on day -34±1. L and H females were placed in cages with running wheels on day -27 and exposed to the foraging task on day 0. Food rationing of R females also began on day 0. Foraging costs of H mice increased by 10% of baseline every other day until day 20 and were then held constant (at 200% baseline). Animals were injected with the novel antigen KLH on day 39±1 and sacrificed on day 49±1. Energy intake is based on grams of food ingested and manufacturer's estimate of energy content of food (line 3 of Table 1). Sample sizes per group were L=8, H=6 and R=7.

RESULTS

Activity, food intake, body mass

Our experimental manipulation affected activity, energy intake and body mass over the course of the experiment (Fig. 1). Chance differences between groups in the baseline phase (Table 2) arose after some animals died or were retrospectively excluded from the study, even though we initially weight-matched the groups [overall mean mass (\pm s.e.m.)=32.6±0.6]. In the baseline phase, R animals also ate significantly less than L and H animals (Table 2). This is most likely because they did not have running wheels in their cages and had lower energy requirements.

Table 2. Responses of female mice to experimental manipulation of foraging costs and food intake

Parameter	Units	Experimental group			H vs L (<i>t</i> -test)		H vs R (<i>t</i> -test)	
		L	H	R	<i>t</i> ₁₂	<i>P</i>	<i>t</i> ₁₁	<i>P</i>
Baseline phase								
Activity	×10 ³ revs day ⁻¹	15.5±2.6	20.9±2.6	—	1.4	0.18	—	—
Energy intake*	kJ day ⁻¹	81.0±2.9	85.3±3.6	65.8±1.6	0.9	0.37	5.2	0.0003
Body mass	g	32.1±0.5	29.8±0.7	30.6±0.9	2.8	0.02	-0.7	0.52
Workload phase								
Activity	×10 ³ revs day ⁻¹	21.0±2.9	24.9±2.4	—	1.0	0.35	—	—
Energy intake*	kJ day ⁻¹	69.5±5.4	52.6±2.6	52.5±2.5	2.5	0.03	0.02	1.00
Body mass	g	33.5±1.3	27.5±1.1	31.0±0.8	3.5	0.005	-2.6	0.02

Female mice experienced low foraging costs (L), high foraging costs (H) or food restriction without foraging (R). Means ± s.e.m. are given for each parameter, calculated over a 7 day interval at the end of each experimental phase (intervals ± 1 day; baseline: -10 to -4; workload: 21 to 27). Samples sizes per group were L=8, H=6, R=7. Results of independent *t*-tests are given (with d.f. shown in subscript). *Energy intake is based on grams of food ingested and manufacturer's estimate of energy content from food (line 3 of Table 1) Bold type highlights significant *P* values.

In order to obtain a constant amount of food, H mice would have needed to double their activity, but they did not do this (Fig. 2). Total activity in workload phase did not differ significantly between L and H females (Table 2), nor did their activity rhythms differ (Fig. 3). L and H mice spent 7.1±0.5 and 8.0±0.3 h per day running, respectively, and ran at maximum speeds of 4.4±0.3 vs 4.4±0.4 km h⁻¹ (mean speeds: L=2.6±0.2, H=2.4±0.3). The two groups did not significantly differ in the amount of time spent running or in their running speeds (*P*>0.1). Although total foraging effort was similar for females facing low and high foraging costs, energy intake was significantly lower for H females (Fig. 1, Table 2). H females (and their R yoked controls) consumed ~25% less energy than L females in the workload phase. H mice did not maintain their body mass and

were significantly lighter than both L and R females in the workload phase (Fig. 1 and Table 2).

Energy metabolism

H mice used significantly less energy than L mice on a daily basis (Table 3). They also used significantly less energy than R mice when removed from the foraging task (RMR, ADMR; Table 3), even though they received the same amount of food at the start of the overnight measurement. We estimated gross energy intake (GEI) during the workload phase from food consumption and food energy content (measured using bomb calorimetry; Table 1). The ratio of DEE:GEI ranged from 0.72 to 0.80 across experimental groups and was significantly higher in H than R mice (Table 3). Estimated surplus energy (S) was significantly lower in H than in R groups (Table 3).

Workload-phase metabolism – both RMR and DEE – was significantly predicted by whole body mass (Fig. 4). Experimental group had an additional, significant effect on DEE (GLM, mass *F*_{1,14}=46.4, *P*<0.0001; group *F*_{2,14}=15.0, *P*=0.0003; model *R*²=0.88; non-significant interaction removed). There was also an effect of group on RMR (GLM, group *F*_{2,15}=6.9, *P*<0.01, *R*²=0.48; non-

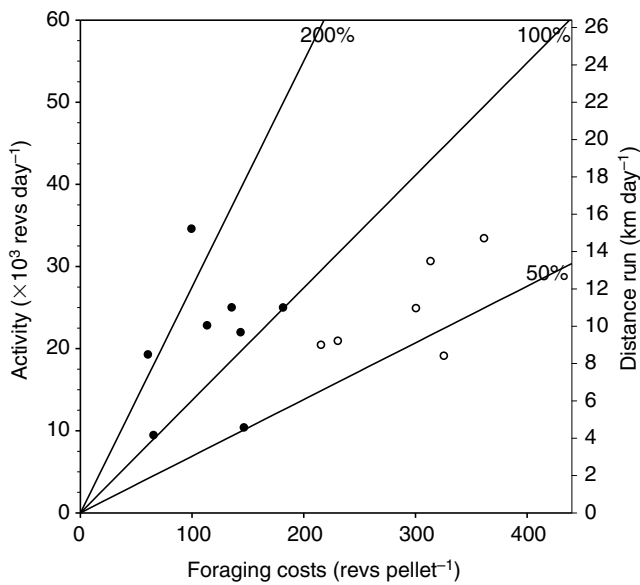


Fig. 2. Wheel-running activity (revs day⁻¹ and km day⁻¹) in relation to workload during the workload phase. Filled circles represent mice facing low foraging costs (individual baseline), and open circles represent mice facing high foraging costs (2× individual baseline). Iso-reward lines show the activity required under different reward rates to obtain a constant amount of food (100% line) double intake (200% line) or reduce intake by half (50% line), ignoring cost of transport. Sample sizes per group were L=8, H=6 and R=7.

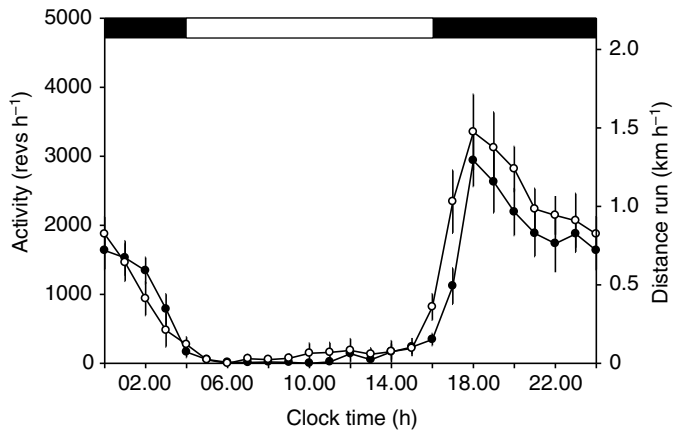


Fig. 3. Circadian rhythm of wheel-running behavior of female mice facing low (L; filled symbols) and high (H; open symbols) foraging costs. Hourly activity averaged over days 21 to 27 of the workload phase showed no differences between groups (for all, *P*>0.1). Sample sizes per group were L=8, H=6 and R=7.

Table 3. Components of energy metabolism for female mice facing foraging costs or food restriction

Parameter	Units	Experimental group			H vs L		H vs R	
		L	H	R	t_{12}	P	t_{11}	P
RQ	–	0.93±0.01	0.96±0.02	0.95±0.02	1.60	0.14	0.34	0.74
RMR	kJ day^{-1}	38.81±1.60	31.63±0.72	35.9±1.72	–3.86	0.003	–2.45	0.04
ADMR	kJ day^{-1}	53.20±0.73	43.16±0.87	48.94±1.94	–8.88	<0.0001	–2.89	0.02
DEE	kJ day^{-1}	69.12±4.94	53.87±2.08	51.09±2.60	–2.68	0.02	0.85	0.42
GEI	kJ day^{-1}	91.74±6.42	67.89±3.05	71.18±3.46	–3.17	0.009	–0.72	0.49
GEI/DEE	–	0.76±0.04	0.80±0.01	0.72±0.02	0.82	0.43	3.49	0.007
S	kJ day^{-1}	3.36±3.64	–0.23±0.77	5.15±1.56	–0.89	0.39	–3.27	0.01
RMR (mass-specific)	$\text{kJ day}^{-1} \text{g}^{-1}$	1.13±0.06	1.12±0.03	1.12±0.05	–0.11	0.91	0.03	0.98
ADMR (mass-specific)	$\text{kJ day}^{-1} \text{g}^{-1}$	1.55±0.06	1.53±0.02	1.53±0.04	–0.29	0.78	0.08	0.94
DEE (mass-specific)	$\text{kJ day}^{-1} \text{g}^{-1}$	1.96±0.08	1.97±0.02	1.59±0.07	0.06	0.96	5.98	0.0002

Female mice experienced low foraging costs (L), high foraging costs (H) or food restriction without foraging (R).

Means ± s.e.m. are given for each parameter in the workload phase (days 21–27). Samples sizes per group were L=7, H=6, R=5. Results of independent t -tests are given (with d.f. shown in subscript).

RQ, respiratory quotient ($V_{\text{CO}_2}/V_{\text{O}_2}$).

RMR and ADMR, resting metabolic rate and average daily metabolic rate, respectively; measured overnight in respirometry chambers at 21°C.

DEE, daily energy expenditure; determined for animals in their home cages using the doubly labeled water method.

GEI, gross energy intake; estimated from food consumption (g day^{-1}) and bomb calorimetry (18.16 kJ g^{-1}).

S, surplus energy; based on an estimate of 79.1% apparent absorption efficiency (Hambly and Speakman, 2005).

Bold type highlights significant P values.

significant effects removed). These effects can be best understood from calculations of mass-specific energy expenditure. Most mass-specific metabolic parameters were virtually identical for H females and both other groups. The exception to this pattern was DEE, which was equal in L and H females, and significantly lower in R than in H mice (Table 3). Overall, H and R mice both saved energy by reducing body mass.

Body composition

We compared the body composition of H females to each of the other two groups on day 50 (Tables 4–6). At this time, H mice had experienced 3 weeks of increasing foraging costs and four weeks of foraging costs at 200% of baseline. The wet masses of all organs were less in H mice than in L mice (Table 4). Wet organ masses were also lower in H than in R mice, but the differences were not as large (Table 4). Organ masses were highly positively correlated within individuals. To analyze differences in overall body composition, we performed a principal components analysis on the correlations between wet organ masses (Table 5). The first three components had Eigenvalues of ≥ 1 , together explaining 74.7% of the total variance in the data. After normalized Varimax rotation, PC1 had high positive loadings for the major metabolically active tissues: the heart, liver, kidneys and brain (Eigenvalue=5.86, percentage variance=53.3). PC1 also had positive loadings for the muscles/skeleton and pelage. PC2 had a high negative loading for the wet empty stomach mass (Eigenvalue=1.27, percentage variance=11.6), and PC3 had high positive loadings for the lungs and intestine (Eigenvalue=1.07, percentage variance=9.8). Comparing PC scores between groups, we found that PC1 and PC3 were significantly reduced in H vs L females (Table 6). High foraging costs, therefore, reduced the size of metabolic organs, musculoskeletal systems, lungs and emptied guts.

All major body components were dramatically lighter in H mice compared to L mice (Table 6). Whole body mass at sacrifice, fresh mass, dry mass, lean dry mass and body fat were all significantly reduced. Body fat was ~77% lower in H females than in L females. The fresh mass of the carcass – which was measured after animals had been exsanguinated and the guts had been emptied – was about

30% lower in H mice than in L mice. Several body components were somewhat lighter in H females than in R animals, but only whole body mass and lean dry mass differed significantly between these groups. H females had 55% less body fat on average than R females, but this difference was not statistically significant. Intestine lengths (measured to the nearest 0.1 cm) did not differ between the groups (mean ± s.e.m.: L=51.9±3.2, H=52.6±3.0, R=55.3±3.0).

Estrous cyclicity

Our experimental manipulation of foraging costs and food intake markedly influenced estrous cyclicity, a measure of reproductive readiness. We restricted all comparisons to females that came into estrus regularly during the baseline phase (L: 6/8, H: 6/6, R: 5/7).

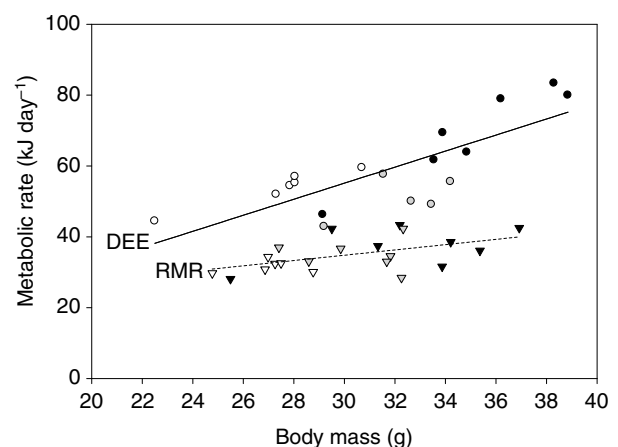


Fig. 4. Relationship between body mass and metabolism during the workload phase. Triangles represent resting metabolic rate (RMR), and circles represent daily energy expenditure (DEE). Body mass significantly predicted both DEE (solid line: $F_{1,16}=26.3$, $P<0.0001$, $R^2=0.62$; $y=2.25x-12.41$) and RMR (dotted line: $F_{1,16}=8.3$, $P=0.01$, $R^2=0.34$; $y=0.80x+10.21$) of mice faced with low foraging costs (black symbols), high foraging costs (open symbols), or food restriction without foraging (grey symbols). Sample sizes per group were L=7, H=6 and R=5.

Table 4. Wet and lean dry masses of body components

Body component	Experimental group		
	L	H	R
Wet mass (g)			
Heart	0.17±0.004	0.13±0.004	0.14±0.004
Liver	1.60±0.07	1.01±0.05	1.18±0.05
Kidneys	0.47±0.02	0.33±0.02	0.38±0.02
Lungs	0.26±0.03	0.19±0.03	0.23±0.02
Brain	0.53±0.02	0.48±0.02	0.51±0.02
Spleen	0.19±0.02	0.12±0.01	0.14±0.01
Uterus, ovaries	0.10±0.02	0.10±0.01	0.10±0.01
Stomach	0.24±0.01	0.21±0.01	0.24±0.01
Intestine	2.40±0.13	1.81±0.13	1.67±0.11
Skin, pelage	4.78±0.44	3.34±0.44	3.91±0.38
Muscles, skeleton	18.27±1.22	12.41±0.93	14.80±0.79
Lean dry mass (g)			
Heart	0.03±0.004	0.02±0.004	0.02±0.004
Liver	0.43±0.02	0.26±0.02	0.30±0.02
Kidneys	0.09±0.01	0.07±0.004	0.08±0.004
Lungs	0.04±0.004	0.03±0.005	0.04±0.004
Brain*	—	—	—
Spleen*	—	—	—
Uterus, ovaries	0.01±0.003	0.01±0.002	0.01±0.002
Stomach	0.07±0.01	0.05±0.01	0.07±0.01
Intestine	0.49±0.05	0.42±0.06	0.38±0.05
Skin, pelage	1.16±0.04	1.00±0.04	1.12±0.04
Muscles, skeleton*	4.75±0.25	3.56±0.16	4.05±0.14

Values are means ± s.e.m. for groups experiencing low foraging costs (L), high foraging costs (H) or food restriction without foraging (R). Samples sizes per group were L=8, H=6, R=7.
*The brain, spleen and a muscle biopsy were removed before drying.

In the workload phase, females facing high foraging costs all ceased to show signs of estrus. H females had a significantly lower proportion of cornified cells than L females (independent *t*-test on arcsin-sqrt transformed data; H vs L: $t_{10}=-5.0$, $P=0.0006$; H vs R: $t_9=-1.9$, $P=0.09$). They were therefore less likely to come into estrus at least once during the final week of the workload phase.
Estrous cyclicity was related to body mass, such that H and R groups had similar mean masses (30.7 and 30.8 g, respectively) on the last day they were observed in estrus. Since the decline in body mass was more rapid in H mice, this similarity in body mass suggests

Table 5. Principle components on correlations between wet masses of body parts

Body component	Factor loadings (wet mass)*		
	PC1	PC2	PC3
Heart	0.8197	-0.1357	0.1597
Liver	0.7279	-0.0965	0.5218
Kidneys	0.7527	0.1171	0.5767
Lungs	0.1949	0.2299	0.7694
Brain	0.8670	0.0299	-0.0783
Spleen	0.6328	0.1245	0.3817
Uterus, ovaries	0.4921	0.4599	0.3818
Stomach	0.1443	-0.9166	0.1065
Intestine	0.1054	-0.2406	0.8130
Skin, pelage	0.6724	-0.1291	0.4820
Muscles, skeleton	0.6753	-0.1975	0.6121

*Factors with Eigenvalues ≥1 were saved. Factor loadings after Varimax normalized rotation are given; values in bold are statistically significant factor loadings.

a body-mass threshold to maintaining fertility in the face of energy limitations. On average, H females were last observed in estrus on day 5, whereas the average last estrous day for R mice was day 19 (the estrous cycle was monitored until day 28). Most H females stopped cycling soon after they were subjected to foraging costs, while food restriction had a more gradual effect on fertility (R group). Treatment group significantly predicted last estrous day overall (Kruskal–Wallis test: $H_{2,17}=7.0$, $P=0.03$), and H females continued cycling longer than L females (Kruskal–Wallis multiple comparisons H vs L: $P=0.04$).

Immunocompetence

An assay of primary immune response performed at the end of the study suggested that the experimental groups differed in antibody production (Fig. 5). Plasma anti-KLH IgG levels at day 10 post-injection did not differ between H and L animals (power analysis: partial $\eta^2=0.32$, $f=0.68$, power=0.73), but were lower in both than in R animals (ANOVA, $F_{2,18}=4.2$, $P=0.03$; LSD *post-hoc* test significant for both L and H vs R). Food restricted mice without running wheels (R), therefore, showed a more robust humoral immune response to a novel antigen than highly active animals

Table 6. Body composition of female mice after experiencing foraging costs or restricted feeding for 50 days

Component	Mass (g)			H vs L (<i>t</i> -test)		H vs R (<i>t</i> -test)	
	L	H	R	t_{12}	<i>P</i>	t_{11}	<i>P</i>
Whole body mass	34.3±1.6	24.1±1.2	27.9±1.0	-10.61	<0.0001	-2.87	0.015
Fresh mass*	29.3±1.8	20.3±1.4	23.6±1.2	-8.66	<0.0001	-2.16	0.054
Dry mass	10.8±1.0	6.2±0.9	7.9±0.8	-5.33	0.0002	-1.91	0.082
Lean dry mass	6.9±0.3	5.3±0.2	5.9±0.2	-7.67	<0.0001	-2.46	0.032
Body fat	3.9±0.8	0.9±0.8	2.0±0.7	-3.67	0.003	-1.47	0.170
PC1	0.68±0.26	-0.75±0.23	-0.13±0.22	-2.81	0.016	-2.16	0.053
PC2	-0.11±0.45	0.28±0.48	-0.12±0.40	0.71	0.491	0.85	0.413
PC3	0.79±0.37	-0.66±0.34	-0.34±0.30	-3.30	0.006	-0.82	0.428

Values are means ± s.e.m. for animals experiencing low foraging costs (L), high foraging costs (H) or food restriction without foraging (R). The H group was separately compared to each other group; results of independent *t*-tests are given (with d.f. shown in subscript). Samples sizes per group were L=8, H=6, R=7.
PC1–PC3 are rotated factor loadings from a principle components analysis on correlations between wet body components (see Table 3).
*Fresh mass was the sum of all carcass components after the animals had been exsanguinated and the guts had been emptied. Some tissue samples were removed before drying. These included the brain, spleen and a muscle biopsy, representing 7.0% of the total fresh mass on average.
Bold type highlights significant *P* values.

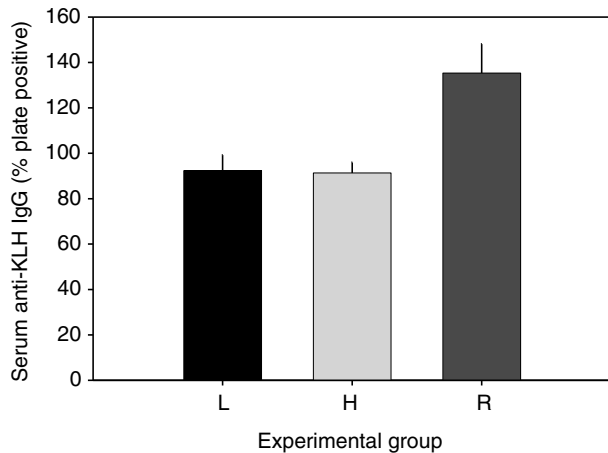


Fig. 5. Serum IgG antibody production in response to the novel antigen, KLH. Tail blood samples were collected 10 days after injection. Mean values are given for female mice under low foraging costs (L), high foraging costs (H), and food restriction without foraging (R). Sample sizes are L=8, H=6 and R=7; bars represent s.e.m.

receiving sufficient food (L) or experiencing restricted feeding (H). The strength of the immune response could not be attributed to body mass or food intake on the day of injection (GLM; mass: $F_{1,19}=0.1$, $P=0.79$; food: $F_{1,19}=1.5$, $P=0.24$).

DISCUSSION

Activity and energy expenditure

Female mice responded to our experimental manipulations with alterations in energy intake and expenditure. Animals subjected to high and low foraging costs obtained different amounts of food, despite showing similar activity levels. Thus, high foraging costs reduced daily gross energy intake and also DEE. Earlier studies manipulating foraging costs per reward with a work-for-food design (Perrigo, 1987; Perrigo and Bronson, 1983; Perrigo and Bronson, 1985; Vaanholt et al., 2007) found similar results but lacked a design feature essential to understanding the underlying process: a control group of food-restricted animals which did not face foraging costs *per se*. The present design allowed us to decouple the most important aspects of high foraging costs: foraging activity and food intake. Our results demonstrate that both the food restriction and the increased activity which result from high foraging costs on a fixed reward schedule contribute to the metabolic and physiological changes observed in those animals.

Our finding that female mice reduced energy expenditure when faced with high foraging costs is similar to the results of other studies using a fixed reward schedule (reviewed by Vaanholt et al., 2007; Wiersma and Verhulst, 2005). However, it does not match the prediction that foraging costs should increase energy expended on food acquisition [see schematic in Wiersma et al. (Wiersma et al., 2005)]. A negative relationship between foraging costs and energy expenditure also differs from observations made by Speakman et al. (Speakman et al., 2003) in free-living short-tailed field voles, which had higher metabolic rates when wintering in poor quality habitat patches. There may be differences in the way animals respond to fixed *versus* variable reward schedules, and thus far, only variable rewards have been shown to elevate DEE (Wiersma et al., 2005; Wiersma and Verhulst, 2005). Disparate effects of fixed and variable reward ratios on energy intake have been attributed to differences in motivation (Fotheringham, 1998). Particularly in

rodents, it remains unclear to what extent limitations on the intensity and duration of activity influence responses to fixed-reward designs.

The female mice in our study ran between 20 000 and 25 000 revolutions per day (~ 9 – 11 km day $^{-1}$) in the workload phase, but activity was not statistically different between groups. Foraging costs did not affect total wheel-running activity. Together with the results of earlier studies, our study provides evidence for limits on sustainable activity levels. Perrigo and Bronson (Perrigo and Bronson, 1983) observed a response qualitatively equivalent to ours in female CF-1 laboratory mice faced with foraging costs between 60 and 225 revs pellet $^{-1}$ (in the same size running wheels). From 60 to 135 revs pellet $^{-1}$, wheel-running activity gradually increased with rising foraging costs. Above 135 revs pellet $^{-1}$, however, mice seemed to reach an activity limit (of just over 15 000 revs day $^{-1}$), regardless of further elevations in foraging costs. There may be sex or strain differences in running behavior. Independent of *ad libitum* activity levels (see Vaanholt et al., 2007), limits to total daily activity may constrain an animal's ability to cope with high foraging costs.

Consequences of foraging costs

Our manipulation had clear effects on energy balance, which were most dramatic in female mice facing high foraging costs. The ratio of DEE:GEI approximates the proportion of consumed energy which would have been assimilated given a net energy balance of zero. The actual assimilation efficiency was probably higher [e.g. 79.1% (Hambly and Speakman, 2005)]. If we assume that animals actually absorbed 79.1% of gross energy intake, we can compare estimated energy balance or energy surplus (S) between groups. Based on this calculation, mice facing high foraging costs would have had no surplus energy or have been in a negative energy balance. By contrast, females facing low foraging costs or food restriction alone were at least 'breaking even'. This conclusion is supported by the fact that the high-cost group lost body mass and showed almost totally depleted fat stores. By contrast, animals in the other groups maintained or increased body mass during the experiment. Similar to Vaanholt et al. (Vaanholt et al., 2007), we found that body composition was dramatically affected by high-cost foraging, and females facing high foraging costs had relatively smaller metabolic organs, muscles/skeleton, and skin/pelage. The effect on body mass was less pronounced in food-restricted animals.

Changes in body mass over time were associated with reductions in energy expenditure. Resting metabolic rate was positively correlated with whole body mass in all groups, and female mice faced with both low and high foraging costs had similar mass-specific metabolic rates in the workload phase. Overall, our RMR estimates of 1.12 kJ g $^{-1}$ day $^{-1}$ for all groups in the workload phase were consistent with Vaanholt's (Vaanholt et al., 2007) estimates of 0.98 kJ g $^{-1}$ day $^{-1}$ for male mice under workload conditions. We did not observe additional reductions in mass-specific energy requirements, however. Vaanholt et al. (Vaanholt et al., 2007) found that male mice on a high workload had reduced mass-specific RMR and DEE compared with baseline conditions. One possible explanation is that although our L group females were not food deprived, they were 'trained' and thereby decreased mass-specific energy requirements. Our data also suggest that high foraging costs force mice to the limit of minimum energy expenditure.

In principle, reducing body mass could have been one component of an energy-saving strategy to prolong survival. Smaller muscles and metabolic organs require less energy, and the cost of transport is reduced with lower body mass (Rezende et al., 2006). Wiersma et al. (Wiersma et al., 2005) suggested that physiological changes made by European starlings (*Sturnus vulgaris*) facing a flight-for-

food paradigm may be energy-saving 'adjustments'. If body mass were not reduced in this context, birds would have been faced with high flight costs, theoretically reducing both food intake and foraging efficiency; alternatively, they would have needed to drastically elevate DEE. Other studies in birds have tested the hypothesis that loss of mass during nestling care can reduce the energetic cost of foraging, thereby potentially increasing reproductive output (e.g. Norbert, 1981). Compensatory mechanisms reduce the impact of nutritional stress on free-living organisms [i.e. small mammals (King and Murphy, 1985)].

Under home cage conditions, minimum energy expenditure may actually have been lower than we estimated from respirometry measurements. Prior studies have suggested that animals facing high foraging costs can reduce resting body temperature (Perrigo and Bronson, 1983; Vaanholt et al., 2007). In a pilot study using the same design as the present one, we found that rectal temperatures of H mice ($35.6 \pm 0.3^\circ\text{C}$) were less than both L ($37.2 \pm 0.2^\circ\text{C}$) and R ($36.4 \pm 0.2^\circ\text{C}$) animals (K.A.S., unpublished data). These differences were small but statistically significant ($N=8$ per group). Measurements of core body temperature in the present study also suggest that animals facing high foraging costs employ daily heterothermy during periods of inactivity.

Trade-offs due to costly foraging

Female mice experiencing high foraging costs ceased to show estrous cyclicity. This response also occurred in some food-restricted females at a later time. Delaying or suppressing the estrous cycle during food restriction (Bronson and Marsteller, 1985) is a strategy for curtailing reproduction at an early stage, and Perrigo and Bronson (Perrigo and Bronson, 1983) found that peripubertal laboratory mice on a workload schedule showed fewer ovulatory cycles. Wild-type female mice (*Mus musculus* and *Peromyscus maniculatus*) faced with high foraging costs were also less likely to become pregnant (Perrigo, 1987). It is interesting that in our study, females on a low workload continued to cycle. This demonstrates that foraging effort does not limit reproductive readiness, but that high foraging costs per reward have a negative influence on fitness prospects.

The results of our immunocompetence assay were intriguing. Female mice experiencing high foraging costs did not show compromised antibody production relative to animals on low foraging costs. The fact that inactive, food restricted mice actually showed higher antibody titers is more difficult to interpret (these data match pilot findings using the same experimental design; K.A.S. unpublished data). From an energetic perspective, one would have expected anti-KLH antibody titers to be lowest in H group females. Primary antibody production after KLH challenge has been shown to be metabolically costly [although it does not induce fever or sickness and does not affect morphological traits such as body mass, adipose tissues, reproductive masses or lymphoid tissues in house mice (Demas et al., 1997)]. Food restriction also reduces immunological memory (Martin et al., 2007) and depresses acute phase response to LPS (a cell-mediated response) in hamsters (Conn et al., 1995) and mice (Matsuzaki et al., 2001). The fact that primary immune response in our experiment was not dependent on energy balance or body fat stores, therefore, is contrary to general findings on the energetics of immunity (reviewed by Demas, 2004). Overall, these results suggest that other factors may be responsible for the effects we observed.

Although voluntary exercise typically enhances immune response [i.e. delayed-type hypersensitivity (Bilbo and Nelson, 2004)], Moraska et al. (Moraska et al., 2000) showed that rats forced to run

on a treadmill mount a compromised primary immune response to KLH. Although L and H females in our experiment could modulate their own activity levels, they had to run in order to receive food pellets. Thus, activity in this setting was not purely voluntary. As Vaanholt et al. (Vaanholt et al., 2007) showed, high wheel running activity under work-for-food conditions increases plasma corticosterone levels, which could be immunosuppressive. Such an effect is probably responsible for the lower immune response of our two foraging groups compared to the inactive, food-restricted group. Our results suggest that immune function may be affected differently in an experimental context linking activity with food intake (i.e. at different foraging costs), then under simple manipulations of energy balance or activity.

CONCLUSIONS

Our results show that energy balance is dramatically altered in small mammals faced with high foraging costs. These extreme changes in energy use, body composition, and other physiological parameters are not stimulated to the same extent by food restriction alone. Manipulations of foraging costs may also have long-term effects. Under poor environmental conditions, animals may obtain less food, while at the same time reallocating energy to activity at the expense of other processes. This may eventually affect rates of senescence (Speakman et al., 2002), possibly yielding results different from studies manipulating food intake alone (i.e. caloric restriction). Ultimately, therefore, studies manipulating the yield of foraging effort can have a broad impact. Research on how animals respond to variation in environmental conditions not only demonstrates ecologically relevant strategies for coping with food scarcity, but it may also lead to a better understanding of physiological processes underlying life history trade-offs.

LIST OF ABBREVIATIONS

ADMR	average daily metabolic rate
DEE	daily energy expenditure
DLW	doubly labeled water
GEI	gross energy intake
KLH	keyhole limpet hemocyanin
RMR	resting metabolic rate
RQ	respiratory quotient
RWA	running wheel activity
S	surplus energy

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